Sirtuin1 Expression and Correlation with Histopathological Features in Retinoblastoma

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Key Words

Sirtuin1 · Retinoblastoma · Histopathology

Abstract

Background: Sirtuin1 (Sirt1) is a member of highly conserved proteins and has been implicated as a tumor promoter as well as a tumor suppressor. One of the mechanisms involves deacetylation of retinoblastoma protein, thereby inhibiting the tumor suppressor function. No study has been reported on the expression of Sirt1 in retinoblastoma. **Methods:** We assessed the expression of Sirt1 in sections of archived tissue blocks of enucleated and exenterated specimens of retinoblastoma patients by immunohistochemistry. The histopathological features were reviewed and correlated with the expression of Sirt1. The effect of Sirt1 expression on survival was also assessed. **Results:** Retrospective data of 94 patients revealed that the median age at presentation was 36 months, with a male:female ratio of 1.9:1. Fifty-one percent of the patients had International Retinoblastoma Staging System (IRSS) stage 1 disease. Of the 94 sections, 89 (95%) expressed Sirt1. Forty-eight percent of the specimens showed grade 3 staining (>75% of the cells), and the intensity was 3+ in 53%. No association between Sirt1 expression and any histopathological feature was noted. Further, Sirt1 expression did not affect the overall and progression-free

survival. **Conclusions:** Sirt1 was expressed in most of the retinoblastoma samples. However, the degree of Sirt1 expression was not associated with any high-risk histopathological feature or survival.

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Introduction

The name ‘sirtuin’ has been derived from *Sir2*, the yeast gene ‘silent mating type information regulation 2 homolog’ responsible for cellular regulation in yeast. Seven sirtuins have been identified in mammals (Sirt1–7), with distinct subcellular localizations and functions [1]. Sirt1 is located in the nucleus, whereas the other sirtuins are located in mitochondria, nucleolus and cytoplasm. The mechanism of action of sirtuins involves their nicotinamide dinucleotide-dependent mono-ribosyltransferase or deacylase activity and a host of substrates including p53 and forkhead box protein O. Sirtuins have been implicated as both tumor suppressor as well as tumor promoter genes [2–5]. Sirt1 has been the most studied sirtuin in carcinogenesis. Sirt1 deacetylates p53 at lysine 382, thereby reducing its transactivation and hence acting as apoptotic factor. However, Sirt1 also plays a key role in the DNA break repair, supporting its tumor suppressor role.

Retinoblastoma protein functions to regulate the G1/S transition of the cell cycle through its interaction with the E2F family of transcription factors [6]. The activity of retinoblastoma protein is regulated predominantly by phosphorylation and dephosphorylation. However, deacetylation of appropriate lysine residues is also known to deactivate the protein [7]. Sirt1 has been reported to bind to the pocket domain of the retinoblastoma protein and to cause its deacetylation, which is nicotinamide dinucleotide-dependent [8].

Since a functional interaction exists between retinoblastoma protein and Sirt1, the latter may be involved in the pathogenesis of retinoblastoma. Overexpression or gain of the function mutation of Sirt1 may lead to the functional loss of retinoblastoma protein and thus to carcinogenesis.

Moreover, Sirt1 inhibitors (Sirtinol, Tenovin-1 and Ex-527) have shown preclinical activity in breast cancer and melanoma by targeting the p53 and PI3K pathway [9, 10]. This class of drugs may provide an effective novel therapy in retinoblastoma if Sirt1 is involved in the pathogenesis.

Based on this hypothesis, we studied the expression of Sirt1 in the archived tissue blocks of enucleated and exenterated specimens of retinoblastoma patients.

Materials and Methods

Study Design

This was a retrospective study conducted at the All India Institute of Medical Sciences, New Delhi, India. The archived tissue blocks of treated retinoblastoma patients were retrieved. The demographic, clinical and survival data of these patients were retrieved from the database at our center. The diagnosis was confirmed by a histopathology review. The review of histopathological features including differentiation, necrosis, calcification, involvement of the optic nerve and its cut end, choroid and other ocular structures was done. The institutional ethics committee approved the study.

Treatment

In patients with IRSS stage I disease, 6 cycles of postenucleation chemotherapy were given as chemoprophylaxis if any high-risk factor was identified in histopathology (invasion of the anterior chamber, iris, ciliary body, sclera, postlaminar optic nerve or massive choroidal invasion); in IRSS stage II disease, 12 cycles of adjuvant chemotherapy along with radiotherapy were administered following enucleation [11]. Stage III tumors were managed with 3 cycles of neoadjuvant chemotherapy followed by enucleation or exenteration, radiotherapy and adjuvant chemotherapy (total 12 cycles) [12]. Metastatic retinoblastoma patients were managed by chemotherapy followed by high-dose chemotherapy with autologous stem cell transplantation in those who had limited metastasis and showed very good response to chemotherapy. The chemo-

therapeutic agents included a combination of vincristine (1.5 mg/m²), etoposide (150 mg/m² for 2 days) and carboplatin (560 mg/m²) administered at 4-week intervals.

Immunohistochemistry

The immunohistochemical detection of Sirt1 protein was performed on formalin-fixed, paraffin-embedded sections (5 µm thick) using rabbit polyclonal Sirt1 antibody (Pierce, Thermo Fisher Scientific, Rockford, Ill., USA). The sections were deparaffinized and rehydrated in ethanol. Antigen retrieval was performed by a heat-based method, i.e. the microwave oven method, in 0.01 mol/l sodium citrate buffer (pH 6.0). Then, the slides were allowed to cool down for additional 5 min in the same buffer. After repeated rinses in tris-buffered saline, the slides were pretreated with hydrogen peroxidase blocking reagent (Thermoquinto) for 5 min. Sirt1 polyclonal antibody (dilution 1:100) was used as the primary antibody for 1 h at room temperature and then at 4°C overnight. After the slides had been washed in tris-buffered saline, secondary incubations were carried out with biotinylated linked secondary antibody and peroxidase-labelled streptavidin (Ultravision Quanto Detection System, Ferment, Calif., USA). Immunoreactivity was visualized using 3',3'-diaminobenzidine peroxidase substrate for 2–3 min, counterstained with hematoxylin and examined by light microscopy. Breast carcinoma was taken as a positive control. Nuclear staining was considered positive for Sirt1 protein.

Classification of Immunohistochemically Stained Patterns

For Sirt1 analysis, tumors were scored by assessment of the proportion and intensity of the stained tumor cells. Positively stained cells were counted in 10 randomly selected fields under ×40 magnification. Intensity was graded as negative (0), weak (1+), moderate (2+) or intense (3+), and the percentage of the stained cells was classified as ≤50% (grade 1), 51–75% (grade 2) or >75% (grade 3). Sirt1 expression of any intensity in >10% cells was considered positive; this was in agreement with one of our previous studies wherein we had used vascular endothelial growth factor antibody in retinoblastoma [13].

Statistical Analysis

The baseline demographic and disease-related variables were summarized using descriptive statistics. Student's t test and χ^2 tests were used for comparing quantitative and qualitative variables in Sirt1-positive and Sirt1-negative patients. Overall survival (OS) and progression-free survival (PFS) of these patients were estimated using Kaplan-Meier survival analysis. Progression was defined as local recurrence or metastasis. Death due to any cause was defined as an event for OS analysis. The impact of Sirt1 on OS and PFS was analyzed using the log-rank test. All statistical tests were done using Stata/SE 13.0 (StataCorp LP, College Station, Tex., USA).

Results

One hundred tissue blocks were retrieved from the archived specimens. Of these, no viable tumors was identified in 6 blocks. Hence, a total of 94 samples were analyzed for Sirt1.

Fig. 1. Expression of Sirt1 protein by immunohistochemical staining (magnification $\times 200$). **a** Negative expression (0); **b** weak staining (1+); **c** moderate staining (2+); **d** intense staining (3+) in poorly differentiated retinoblastoma.

Sirt1 was found to be positive in 89 (95%) and negative in 5 (5%) samples (fig. 1). Intense staining (3+) was observed in 50 (53%) samples, whereas 45 (48%) samples showed grade 3 staining (online suppl. table; see www.karger.com/doi/10.1159/000439594 for all online suppl. material). The baseline characteristics and treatment-related factors of these 94 retinoblastoma patients are shown in table 1.

Correlation with the Histopathological Features

For comparison, expression was classified as grade 3 versus the rest. There was no difference in histopathological features between these 2 groups (table 2).

Effect on Survival

The median follow-up of this group of patients was 59 months (95% confidence limits, 50–63). There was no effect of the degree of Sirt1 expression (grade 3 vs. rest) on OS or PFS [5-year OS: 93% (81–98) vs. 86% (72–93), $p = 0.23$; 5-year PFS: 89% (75–95) vs. 88% (75–94), $p = 0.85$]. In a subgroup analysis of 46 extraocular and metastatic retinoblastoma patients, no difference was observed between the two groups [5-year OS: 94% (63–99) vs. 83% (64–93), $p = 0.33$; 5-year PFS: 87% (68–95) vs. 81% (52–93), $p = 0.66$] (online suppl. figure).

Discussion

This study found that Sirt1 was positive in 95% of the samples. Increased Sirt1 expression has been reported previously in colon, skin, breast and prostate cancer and leukemia [14–17]. However, decreased Sirt1 mRNA expression has been reported in other studies in prostate and bladder carcinoma, glioblastoma and ovarian cancer [18]. A study elucidated that Sirt1 can deactivate retinoblastoma protein by deacetylation [8]. We hypothesize that the high frequency of Sirt1 expression in retinoblastoma may suggest a role of Sirt1 in the pathogenesis by deacetylation and hence inactivation of retinoblastoma protein.

Sirt1 positivity was associated with a higher tumor size and TNM stage in non-small cell lung cancer and uterine cancer patients [18–20]. Contrary to this, Sirt1 expression was associated with a lower tumor stage in ovarian serous adenocarcinoma and oral squamous cell cancer [20, 21]. In this study, Sirt1 expression did not predict the stage or any high-risk histopathological features.

Likewise, Sirt1 expression was associated with a shorter OS in lung cancer and diffuse large B-cell lymphoma and with prolonged OS in ovarian serous adenocarcinoma [21–23]. However, in our study, no effect of Sirt1 expression was observed either in the entire cohort or in a

Table 1. Baseline and treatment-related factors of the patients

	Sirt1 <grade 3 (n = 49)		Sirt1 grade 3 (n = 45)	p value
Age at diagnosis, months				0.55
Median	36	36	30	
Mean	39±29	41±27	37±31	
Range	2–192	2–144	3–192	
Sex				0.56
Male	62 (66)	31 (63)	31 (69)	
Female	32 (34)	18 (37)	14 (31)	
Disease				0.79
Unilateral	66 (70)	35 (71)	31 (69)	
Bilateral	28 (30)	14 (29)	14 (31)	
IRSS Stage				0.1
1	48 (51)	19 (39)	29 (64)	
2	3 (3)	2 (4)	1 (2)	
3	38 (40)	25 (51)	13 (29)	
4	5 (5)	3 (6)	2 (4)	
Surgery				0.61
Enucleation	87 (93)	44 (90)	43 (96)	
Exenteration	7 (7)	5 (10)	2 (4)	
Chemotherapy				0.61
No	7 (7)	3 (6)	4 (9)	
Yes	87 (93)	46 (94)	41 (91)	0.1
Radiotherapy				
No	48 (51)	21 (43)	27 (60)	
Yes	46 (49)	28 (57)	18 (40)	

Values are n (%), unless otherwise specified. The percentages have been rounded-off to the nearest number and hence the total may not be 100.

limited group of extraocular and metastatic retinoblastoma.

Sirt2 and Sirt6 have recently been reported to be expressed in all 18 retinoblastoma blocks with varying intensity [24]. This study along with our study points to the functional relation between Sirt proteins and retinoblastoma and requires future functional studies.

To conclude, this study demonstrated that more than 95% of the retinoblastoma specimens expressed Sirt1. Despite this high frequency of expression, the degree of expression was not associated with any histopathological features or survival. The near uniform expression of Sirt1 in retinoblastoma specimens may suggest a pathophysiological and therapeutic role. Whether Sirt1 inhibitors will be effective in retinoblastoma needs further evaluation.

Table 2. Correlation of histopathological features with Sirt1 expression

	Sirt1 <grade 3 (n = 49)		Sirt1 grade 3 (n = 45)	p value
Differentiation				0.87
Good	6 (50)		6 (50)	
Poor	43 (52)		39 (48)	
Necrosis				1
No	3 (60)		2 (40)	
Yes	46 (52)		43 (48)	
Optic nerve resected margin or postlaminar involvement				0.8
No	36 (53)		32 (47)	
Yes	13 (50)		13 (50)	
Choroid invasion				0.29
No	26 (58)		19 (42)	
Yes	23 (47)		26 (53)	
Anterior chamber involvement				0.54
No	39 (51)		38 (49)	
Yes	10 (59)		7 (41)	
Iris involvement				0.73
No	45 (53)		40 (47)	
Yes	4 (44)		5 (56)	
Ciliary body involvement				0.77
No	43 (53)		38 (47)	
Yes	5 (46)		7 (54)	
Scleral invasion				0.64
No	41 (53)		36 (47)	
Yes	8 (47)		9 (53)	

Values are presented as n (%). The percentages have been rounded-off to the nearest number and hence the total may not be 100.

Statement of Ethics

All procedures involving human participants performed in the study were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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